

## MECHANISM OF ACTION AND BIOCHEMICAL EFFECTS OF NITRIC OXIDE (NO<sup>•</sup>)

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**Abstract.** Nitric oxide (NO<sup>•</sup>) synthesized in endothelial cells, from the terminal guanidino nitrogen atom of L-ARG, by means of NO-synthase (NOS), activates guanylyl-cyclase in smooth muscle cells and platelets, increasing the levels of the intracellular messenger cyclic guanylyl phosphate (GMPc). This phenomenon produces smooth muscle relaxation and platelet aggregation inhibition, presumably by reduction of the intracellular free Ca<sup>2+</sup> concentration. The endothelial vasodilator prostacyclin causes the same effects through adenylyl-cyclase activation, which increases intracellular level of AMPc. The biological activity of NO<sup>•</sup> may be modified by oxygen-derived reactive species, such as anion superoxide (O<sub>2</sub><sup>•-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical (OH<sup>•</sup>), contributing to regulate the vascular tone. NO<sup>•</sup> may possess both cytoprotective and cytotoxic properties, depending on the amount and the isoform of NOS. NO<sup>•</sup> may regulate hepatic metabolism directly by causing alterations in hepatocellular metabolism and function, or indirectly as a result of its vasodilator properties.

**Key words:** nitric oxide (NO<sup>•</sup>), oxygen-derived reactive species, peroxynitrite (ONOO<sup>-</sup>), cytoprotective or/and cytotoxic properties

**Rezumat.** Oxidul nitric (NO<sup>•</sup>) sintetizat în celulele endoteliale, din atomul de N guanidino terminal al L-ARG, prin intermediul enzimei NO-sintetaza (NOS), activează guanilat-ciclaza din celulele musculare netede și plachete, conducând la creșterea nivelului mesagerului intracelular guanozil-monofosfat ciclic (GMP<sub>c</sub>). Acest proces va produce relaxarea mușchilor netezi și inhibarea agregării plachetare, probabil prin reducerea concentrației intracelulare a Ca<sup>2+</sup> liber. Vasodilatatorul endotelial prostaciclina (PGI<sub>2</sub>) cauzează același efect, activând însă adenilat ciclaza, care va crește nivelul intracelular al mesagerului secund AMP<sub>c</sub>.

Activitatea biologică a NO<sup>•</sup> poate fi modificată prin acțiunea speciilor reactive derivate de la O<sub>2</sub>, cum ar fi anionul superoxid (O<sub>2</sub><sup>•-</sup>), peroxidul de hidrogen (H<sub>2</sub>O<sub>2</sub>) și radicalul hidroxil (OH<sup>•</sup>), contribuind la reglarea tonusului vascular. NO<sup>•</sup> poate avea un rol atât citoprotectiv cât și citotoxic, depinzând de concentrație și de izoforma NOS. NO<sup>•</sup> poate regla în mod direct metabolismul hepatic cauzând alterări în metabolismul și funcția hepatocelulară, sau indirect, ca rezultat al proprietăților sale de vasodilatator.

**Cuvinte cheie:** oxidul nitric (NO<sup>•</sup>), specii reactive derivate din O<sub>2</sub>, peroxinitrit (ONOO<sup>-</sup>), proprietăți citoprotective și/sau citotoxice

### INTRODUCTION

Nitric oxide was first identified in 1987 (Ignarro, 1987) bringing him two years later the Nobel Prize in Science

(1). Nitric oxide is made at various sites in the body and it performs important function in many systems:

- **Blood pressure:** nitric oxide plays an important role in the maintenance of healthy blood pressure and, in turn, cardiovascular health.

- **Heart:** when arteries become clogged, they produce less nitric oxide than normal. The treatment with nitroglycerin produces an increased level of nitric oxide, thus widening blood vessels and increasing blood flow.

- **Infections:** huge quantities of nitric oxide are produced in whole blood cells to kill invading bacteria and parasites.

- **Shock:** if too much nitric oxide is produced, it can dilate blood vessels dropping the blood pressure.

- **Lungs:** inhalation of nitric oxide gas has been effective in treating some intensive care patients including infants with lung disorders.

- **Nervous system:** when nitric oxide is formed in nerve cells, it can stimulate the brain and modulate many functions, from behavior to gastrointestinal activity.

- **Cancer:** white blood cells use nitric oxide to defend the body against tumors. Research are running now to investigate whether it can be used to stop the tumor growth.

Taking these into account it is evident that nitric oxide has many clinical, biochemical and public health implications.

On the other hand, NO is a common air pollutant from combustion sources and the exhaled NO was measured for environmental and occupational studies to test the airway dysfunction and related-diseases (2,3). NO also can be converted to toxic metabolites

such as peroxynitrite, which convert cholesterol-carrying lower density lipoproteins to a form that contributes to atherosclerotic plaque formation (2). The involvement of nitric oxide in different pathological processes, such as atherosclerosis, diabetes, ischemia and reperfusion, or in inflammatory process was outlined until now (4).

In this context, this presentation aim to underline some biochemical aspects of nitric oxide, as both cause and effect of real interest in public health.

### 1. Mechanism of action of NO

Nitric oxide is released by the endothelial cells through various stimuli, such as 5-OH-tryptamine, acetylcholine, thrombin, A32187 calcium ionophor, arachidonic acid, changes in arterial pressure, electric stimulation etc., either as NO<sup>•</sup>, or bound to a -SH group-containing carrier molecule (e.g. L-cys) that stabilizes NO<sup>•</sup> release (5,6,7).

Once released, NO<sup>•</sup> activates the guanylate cyclase in the smooth muscle cells and platelets rising the level of intracellular messenger cGMP. This rise causes smooth muscle relaxation and platelet aggregation inhibition, presumably by a decrease in intracellular Ca<sup>2+</sup> concentration (7,8).

The endothelial vasodilator prostacyclin (PGI<sub>2</sub>) causes the same effect by the activation of adenylyl-cyclase that increases the intracellular level of adenosyl monophosphate (AMP) (fig. 1).

The same mechanism seems to be involved in NO-induced platelet aggregation inhibition.

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NO<sup>•</sup> is less stable than other endothelial vasodilators, such as prostacyclin. The latter has a half-time of about 3 minutes and is rapidly converted to the inactive compound 6-keto-PG-F<sub>1α</sub>. Both endothelial agents cause platelet aggregation inhibition and vessel relaxation, but through different mechanisms (6), namely the rise in cGMP level in platelets and central nervous system and rise in intracellular cAMP level, respectively. Moreover, NO<sup>•</sup> released by neutrophils and other white cells enhances the platelet antiaggregant effect of endothelial PGI<sub>2</sub> (6).

NO<sup>•</sup> can bind to oxi-Hb and Fe-SH complexes of other proteins, thus modulating the activity of many hepatic enzymes (9,10). Studies on isolated hepatocytes (11) showed that, phosphorylation of IP<sub>3</sub> (cGMP-dependent protein kinase receptor) increases its sensitivity to P-inositol by the release of free intracellular Ca<sup>2+</sup>. Another hypothesis suggests that endothelium produces a hyperpolarizing factor that may induce vasodilatation. It has been demonstrated that NO<sup>•</sup> induces hyperpolarization through the opening of K<sup>+</sup> channels in the smooth muscles (12). It is not clear yet if this is facilitated by the rise in intracellular cGMP concentration, but the resulting hyperpolarization may represent an

important aspect of NO<sup>•</sup> action in other cell types. As an example, the activation of soluble guanylate cyclase was noticed in the hepatocytes stimulated to produce NO<sup>•</sup> by the exogenous introduction of NO<sup>•</sup> directly in culture (12).

### **2. Direct effects of NO<sup>•</sup>**

#### **2.1. NO<sup>•</sup> - cytotoxic and/or cytoprotective agent**

NO is an effector molecule essential in the antitumoral, antimicrobial, and antiviral action of activated immune cells (5,13). Cytokine-induced NO<sup>•</sup> loses the “mask” of harmless and changes into a true cytotoxic agent, both itself and through the interaction products with other reactive species generated by activated macrophages (14). NO<sup>•</sup> induces in target cells some metabolic dysfunctions, such as: inhibition of respiratory chain and Krebs' cycle, inhibition of DNA synthesis, massive loss of intracellular iron with alteration of ferritin and transferrin receptors function, inhibition of glycolysis and -Fe-S group-containing mitochondrial enzymes (6,13,14), metabolic disturbances that might culminate with the death of target cells (fig. 2).

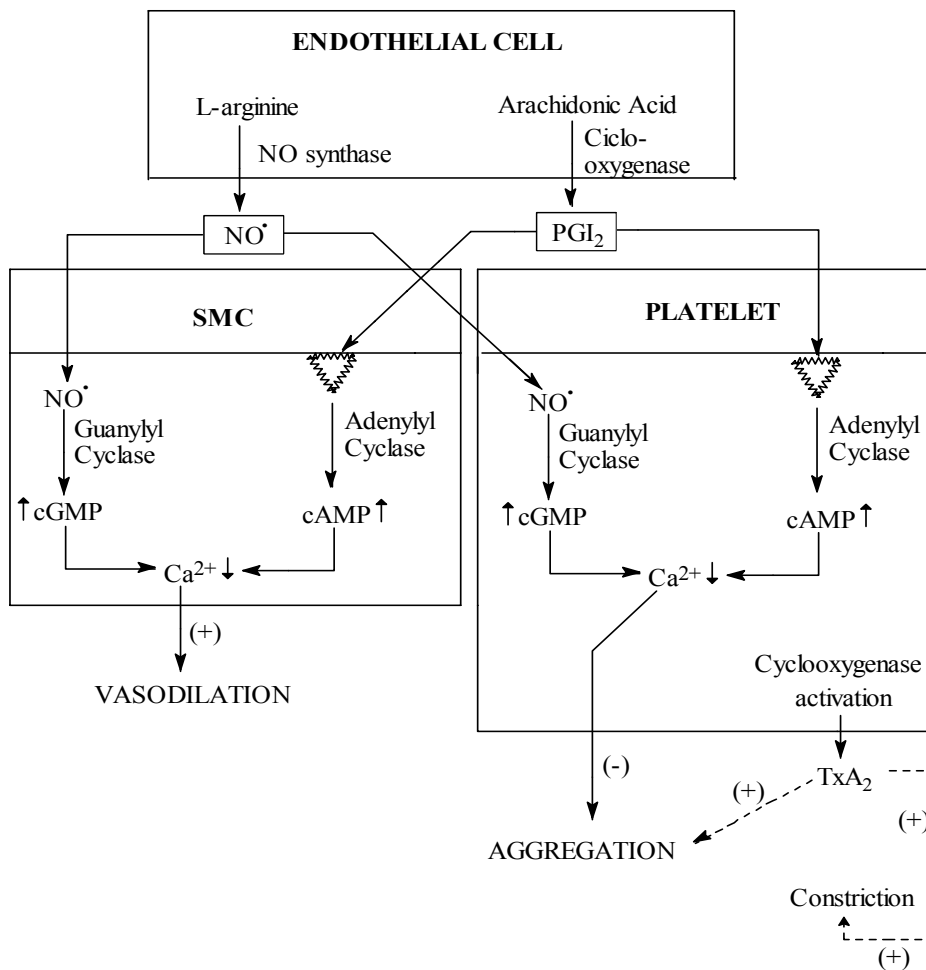


Figure 1. Mechanisms of action of nitric oxide (NO) (9)

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**IS NO CYTOPROTECTIVE OR CYTOTOXIC?**

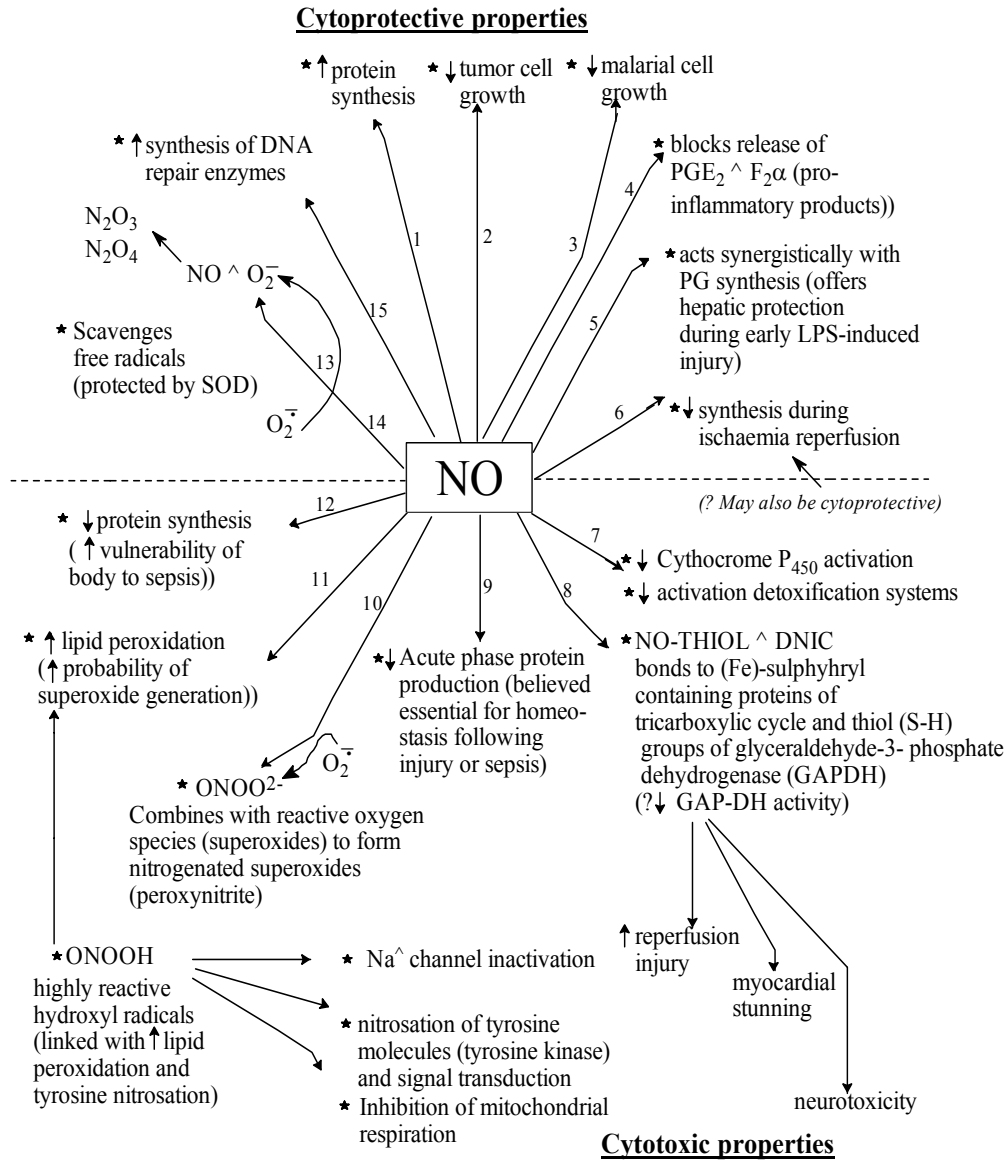


Figure 2. The cytoprotective and/or cytotoxic properties of NO\*(9)

The cytotoxic potential of peroxynitrite anion  $\text{ONOO}^-$  (15,16,17), major cytotoxic agent resulting from  $\text{NO}^\bullet + \text{O}_2^\bullet$  reaction, is mainly due to direct or indirect oxidation of -SH groups in the structure of proteins and some nonprotein compounds, resulting in

disturbed metabolic pathways and membrane functions (18,19). The oxidation of -SH groups in Cys and GSH exhausts an important oxidation mechanism, that of scavenger of  $\text{O}_2$  reactive species (SRDO) (13,20) (table 1).

**Table 1. Generation of SRDO and SRDN in the inflammatory cells of the immune system (13)**

Generation of SRDO	Generation of SRDN
1. Glucose metabolization produces NADPH (pentose phosphates pathway)	NO synthesis under the action of iNOS
2. NADPH-oxidase (plasma-membrane) reduces $\text{O}_2$ to anion superoxide:  $\text{NADPH} + \text{H}^+ + 2 \text{O}_2 \rightarrow \text{NADP}^+ + 2 \text{H}^+ + 2 \text{O}_2^\bullet$	NO interacts with $\text{O}_2^\bullet$ forming $\text{ONOO}^-$ anion, which in the presence of transient metals can generate nitronium ions ( $\text{NO}_2^+$ )
3. SOD decomposes $\text{O}_2^\bullet$ to $\text{H}_2\text{O}_2$ :  $2 \text{O}_2^\bullet + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	$\text{ONOO}^-$ peroxynitrite anion protonates in acid environment forming peroxynitrous acid, which homolythically splits into 2 “virulent” radicals, $\text{OH}^\bullet$ and $\text{NO}_2^\bullet$ $\text{ONOO}^- \xrightarrow{\text{H}^+} \text{ONOOH} \rightarrow \text{OH}^\bullet + \text{NO}_2^\bullet$
4. In the presence of $\text{Fe}^{2+}$ (with catalytic role), $\text{H}_2\text{O}_2$ reacts with $\text{O}_2^\bullet$ generating the $\text{OH}^\bullet$ radical, highly reactive and with a non-discriminating action:  $\text{H}_2\text{O}_2 + \text{O}_2^\bullet \xrightarrow{\text{Fe}^{2+}} \text{OH}^- + \text{OH}^\bullet + \text{O}_2$  SRDO = $\text{O}_2$ -derived reactive species	SRDN = nitrogen-derived reactive species

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Cytokine-induced NO<sup>•</sup> production takes also place in hepatocytes, fibroblasts, and endothelial cells in response to various aggressions, thus suggesting the importance of N-derived reactive species (SRDN) in the cytotoxic activity of some cell types other than activated macrophages and neutrophils (table 1) (13,20).

NO<sup>•</sup> is also involved in the cytotoxic effect of macrophages and neutrophils (7) possessing NOS (21) and the ability of synthesizing NO<sup>•</sup> (12,22-24).

NO<sup>•</sup> contributes to macrophage-mediated immune function, and especially to the so called "non specific host defense", such as killing of tumor cells, antimicrobial response or rejection of implanted organs (6).

NO<sup>•</sup> plays a major role in the *in vitro* killing of endothelial cells by neutrophils. Activated neutrophils may generate O<sub>2</sub><sup>•</sup> (25), which together with NO<sup>•</sup> induce the generation of the highly reactive toxic radical OH<sup>•</sup> that might be ultimately responsible for the death of endothelial cells (6).

Liver Kupffer (K) cells are also capable of synthesizing NO<sup>•</sup> (5,11), acting as liver macrophages (as anatomic location) and like these ones being phagocytic and able to ingest microorganisms and bacterial toxins. The ratio K cells/hepatocyte count may be important in the production of NO<sup>•</sup> and hepatocyte cytotoxicity. The ratio is high following endotoxins or immunostimulating infections (26). When exposed to inflammatory stimuli, such as LPS (endotoxin) and  $\gamma$ -interferon, K cells release tumoral

necrosis factor (TNF) and IL-1, which may then stimulate iNOS synthesis in hepatocytes. The simultaneous release of numerous cytokines might act synergically for inducing hepatic iNOS (26).

The complex action of K cell inflammatory mediators, including TNF, IL-1, IL-6 and NO<sup>•</sup>, can not be determined *in vivo*, and it is still unclear whether they exert or not a cytoprotective and/or cytotoxic action (fig. 2).

NO<sup>•</sup>, a superoxide cell itself, may form N<sub>2</sub>O<sub>3</sub> and N<sub>2</sub>O<sub>4</sub> in the presence of O<sub>2</sub><sup>•</sup> and, within this context, can be considered cytoprotective. The cytotoxic properties of NO<sup>•</sup> associated with its ability to form ONOO<sup>-</sup> produce a highly reactive compound, ONOOH, which amplifies lipid peroxidation and increases the probability of other reactive radicals generation (7,18,24).

S. Mondoca et al. (7) emphasize that the ability of NO<sup>•</sup> to react with Fe-nitrosil and -SH group-containing ligands forming nitrosil-iron-cys (DNIC) and nitrosothiols (NO-THIOL) (fig.2) is related to a reduced GAP-DH activity (6,9).

To conclude, the potential of NO<sup>•</sup> to exert a cytotoxic and/or cytoprotective action seems to be related to its ability of interacting with other nearby molecules and to the produced amount.

**2.2. The vasoconstrictive and vasodilator responses of NO<sup>•</sup> require the presence of anion superoxide O<sub>2</sub><sup>•</sup>, OH<sup>•</sup> and H<sub>2</sub>O<sub>2</sub>**

**a) Vasoconstrictive responses**

It is known the fact that  $O_2^{\bullet -}$  is involved in endothelial cell death following exposure to  $H_2O_2$  or forbol esters-activated neutrophils.

Endothelial cells contain xanthine-oxidase (XO) in a 2:1 xanthine DH/xanthine oxidase ratio, but in contact with activated neutrophils this ratio becomes 1:2. This conversion process is irreversible and cannot be blocked by the presence of superoxide dismutase (SOD) and catalase (CAT) (27).

Increased endothelial XO activity leads to  $O_2^{\bullet -}$  generation, which can reduce intracellular  $Fe^{3+}$ , thus allowing the FENTON reaction to take place and  $OH^{\bullet}$  to form. Eventually, this free radical seems to be responsible for the damaged endothelial cells.

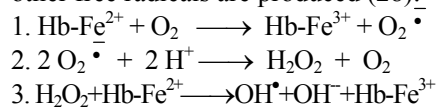
$O_2^{\bullet -}$  anion inactivates  $NO^{\bullet}$ , which causes vasoconstriction. The relaxation is mediated by the formation of  $ONOO^-$ , which seems to stimulate soluble guanylate cyclase in the smooth muscle cells and by the production of a soluble superoxide that mediates the relaxation factor ( $O_2^{\bullet -}$  - RF), which causes hyperpolarization by the opening of glibenclamid-sensitive  $K^+$  channels.

EDRF inactivation by  $O_2^{\bullet -}$  was recognized in 1985, even before the fact that EDRF is actually  $NO^{\bullet}$  was demonstrated (7) (fig. 3).

Some specific enzymes, reaction and different compounds are either stimulators (+) or scavengers (-) of  $O_2^{\bullet -}$ ,  $OH^{\bullet}$ , and  $H_2O_2$  production.

$O_2^{\bullet -}$  anion can reduce oxidizing substances, as well as other agents,

such as catecholamines or Hb, which in their turn can inactivate the transient EDRF/ $NO^{\bullet}$ . Thus,  $NO^{\bullet}$  production can be a common mechanism of EDRF/ $NO^{\bullet}$  inactivation by these agents (3, 5, 18). With Hb, during oxiHb ( $Fe^{2+}$ ) autooxidation to metHb ( $Fe^{3+}$ ),  $O_2^{\bullet -}$  anion and secondary, other free radicals are produced (28):



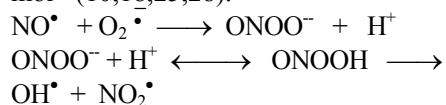
The fact that  $O_2^{\bullet -}$  generated by other sources mediates the vasoconstrictive responses by  $NO^{\bullet}$  inactivation was noticed.

YANG et al. (27) showed that the exposure of rat aortic arteries to X-XO induces a mild basal contraction and a strong noradrenalin-mediated contraction able to generate  $O_2^{\bullet -}$ , although these effects can be blocked by previous SOD treatment of the area.

**b) Vasodilator responses**

$H_2O_2$  causes vasodilatation by stimulating  $NO^{\bullet}$  release and/or activation of soluble guanylate cyclase. The vessel relaxation effect of  $OH^-$  radical is due to its ability to activate soluble guanylate cyclase (7,9).

The vasodilator responses also involve the presence of  $O_2^{\bullet -}$ , by the formation of  $ONOO^-$  peroxynitrite, resulting from the reaction between  $NO^{\bullet}$  and  $O_2^{\bullet -}$ , at a constant rate of  $6.7 \times 10^9 L s^{-1} mol^{-1}$  (10,18,25,28).



When protonated,  $ONOO^-$  can lead to  $NO_2^{\bullet}$  and  $OH^{\bullet}$  formation. Peroxynitrite



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is a powerful oxidant that can react with a great variety of compounds, from deoxyribose and lipids to -SH groups and methionine residues of proteins (10,25,29).

Another function of this agent was described by D. KU and S. LIU (14). They showed that the exposure of

human and canine coronary arteries to ONOO<sup>-</sup> promotes a long-lasting relaxation via a mechanism having properties similar to those exerted by NO<sup>•</sup> (fig. 3).

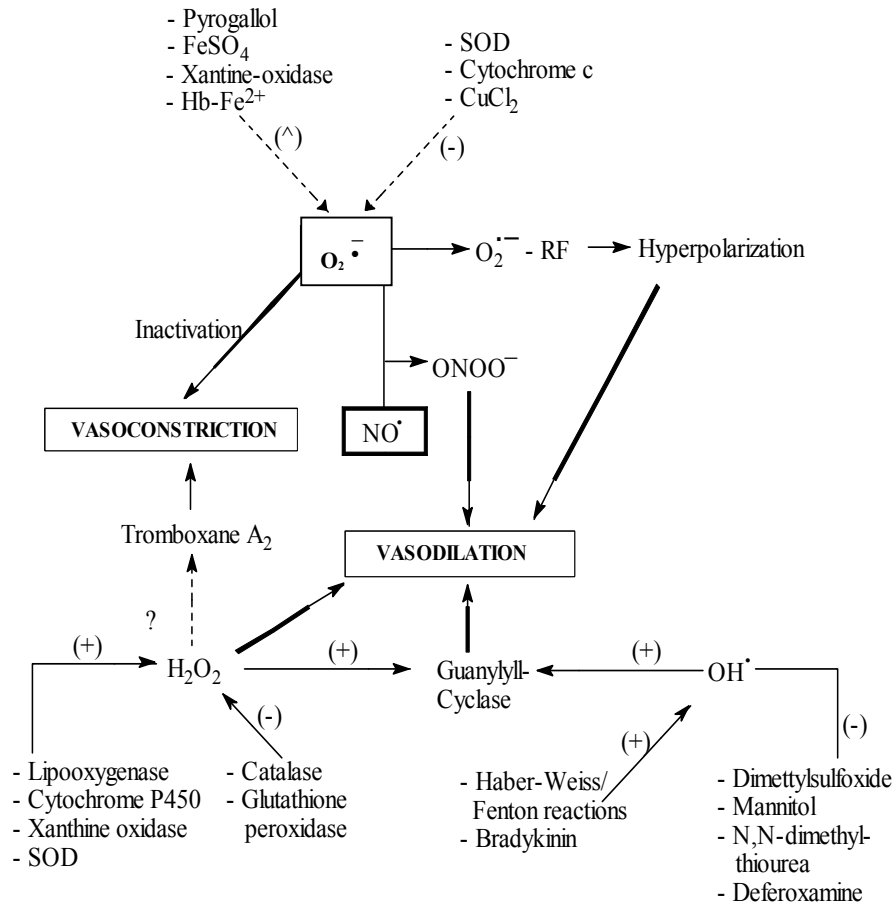


Figure 3. Mechanisms of vasodilator and vasoconstrictor responses induced by O<sub>2</sub><sup>•-</sup>, OH<sup>•</sup> and H<sub>2</sub>O<sub>2</sub> (6)

Similar results have been noticed in bovine lung arteries, where ONOO<sup>-</sup>-induced relaxation seemed to be due to nitrosylated -SH groups in the tissues, which later on release NO<sup>•</sup>. ONOO<sup>-</sup>-induced relaxation seems to involve the stimulation of soluble guanylate cyclase, although it is inhibited by methylene blue and LY-83583. These agents act as inhibitors of guanylate cyclase stimulation via an increased O<sub>2</sub><sup>•</sup> production. Consequently, the free radicals in the smooth muscles are capable of modulating the NO<sup>•</sup>-mediated relaxation of ONOO<sup>-</sup> (10,18,19).

Another mechanism involved in O<sub>2</sub><sup>•</sup>-mediated vasodilatation consists in the production of an endothelium-derived stable relaxation factor, different from EDRF, released through acetylcholine (6), its vasodilator effect not being related to a rise in cGMP level in the smooth muscles. It was demonstrated that this O<sub>2</sub><sup>•</sup>-mediated relaxation factor inhibits, by anoxia-reoxygenation, the decline in viability of isolated rat cardiac myocytes in a similar way as chromokalin (K<sup>+</sup> channel opener) (6). It seems that this O<sub>2</sub><sup>•</sup>-mediated relaxation factor causes vasodilatation by the opening of glibenclamide-sensitive K<sup>+</sup> channels, causing smooth muscle hyperpolarization and relaxation (6).

It is now known that NO<sup>•</sup> can modulate the tone of the portal vein and hepatic artery, that can alter the liver metabolism (9,24).

As many vasoactive substances (autocoids) can exert their action via NO<sup>•</sup> release, it is likely that the

modulating action upon hepatic vascular tone to undergo changes in the presence of some conditions that directly influence NO<sup>•</sup> release and synthesis in the liver (13,28).

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